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Cytolethality of Glutathione Conjugates with Monomethylarsenic or Dimethylarsenic Compounds

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Arsenicals are known to be toxic and carcinogenic in humans. Inorganic arsenicals are enzymatically methylated to monomethylarsonic acid ($MMAs^V$) and dimethylarsinic acid ($DMAs^V$), which are the major pentavalent methyl arsenic metabolites. Recent reports indicate that trivalent methyl arsenicals are produced through methylation of inorganic arsenicals and participate in arsenic poisoning. Trivalent methyl arsenicals may be generated as arsenical–glutathione conjugates, such as monomethylarsonous diglutathione ($MMAs^{III}DG$) and dimethylarsinous glutathione ($DMAs^{III}G$), during the methylation process. It has been well known that reduced glutathione (GSH) reduces $MMAs^V$ and $DMAs^V$ *in vitro*, and produces $MMAs^{III}DG$ and $DMAs^{III}G$. Some studies have shown that exogenous GSH increased cytolethality of $MMAs^V$ and $DMAs^V$ *in vitro*, while other studies have suggested that exogenous GSH decreased them. In this study, we examined the true effects of exogenous GSH on the cytolethality of $MMAs^V$ and $DMAs^V$ by investigating reactions between various concentrations of $MMAs^V$ or $DMAs^V$ and GSH. GSH significantly increased the cytolethality and cellular uptake of pentavalent methyl arsenicals when GSH over 25 mM was pre-incubated with mM levels of arsenicals, and this cytolethality might have been caused by arsenical–GSH conjugate generation. However, GSH at less than 25 mM did not affect the cytolethality and cellular uptake of pentavalent methyl arsenicals. These findings suggest that high concentrations of arsenicals and GSH are needed to form arsenical–GSH conjugates and to show significant cytolethality. Furthermore, we speculated that $MMAs^{III}DG$ and $DMAs^{III}G$ may separate into trivalent methyl arsenicals and glutathione, which are then transported into cells where they show significant cytolethality.

Key words arsenic; glutathione (GSH); dimethylarsinic; monomethylarsonic; GSH conjugate

Arsenic is a metalloid element that is widely distributed in the environment as a natural component of soil and in water as inorganic trivalent (arsenite) or pentavalent (arsenate) forms,[1] and its toxicity has been known since ancient times. In Asia and the Americas, chronic arsenic poisoning has occurred as a result of the consumption of high levels of arsenic-contaminated well water. Epidemiological studies have provided clear evidence that inorganic arsenicals are a human carcinogen with target sites including liver, skin, lung, kidney and urinary bladder.[2] However, the mechanism of arsenic-induced impediment is not clear. On the other hand, inorganic arsenite has emerged as a potent chemotherapeutic agent with remarkable efficacy for certain human cancers, such as acute promyelocytic leukemia.[3,4] It would thus appear that environmental and iatrogenic exposure to arsenic will continue to be common.

In humans and numerous experimental animals, pentavalent arsenate is rapidly reduced to trivalent arsenite.[5] Subsequently, it is enzymatically methylated into organic arsenicals, such as monomethylarsonic acid ($MMAs^V$) and dimethylarsinic acid ($DMAs^V$).[6] $MMAs^V$ and $DMAs^V$ are the major organic pentavalent arsenic metabolites in human urine after exposure to inorganic arsenicals.[5,6] It is believed that methylation of inorganic arsenicals results in a reduction in general toxicity, as indicated by their increased *in vivo* lethal dose in 50% of a population (LD_{50}) and *in vitro* lethal concentration in 50% of a population (LC_{50}).[7,8] However, recent studies have increasingly suggested that the methylation of inorganic arsenicals is not a universal detoxification mechanism. Some researchers have reported that trivalent methyl arsenicals, such as monomethylarsonous acid ($MMAs^{III}$) and dimethylarsinous acid ($DMAs^{III}$), were found in urine collected from people who had been exposed to high concentrations of inorganic arsenicals,[9,10] and that synthetic trivalent methyl arsenicals, such as monomethylarsine oxide ($MMAs^{III}O$) and iododimethylarsine ($DMAs^{III}I$), are more cytotoxic *in vitro* than inorganic arsenicals and pentavalent methyl arsenicals.[11] Trivalent methyl arsenicals are thought to be generated as arsenical–glutathione conjugates, such as mono-methylarsonous diglutathione ($MMAs^{III}DG$) and dimethylarsinous glutathione ($DMAs^{III}G$), in the human body.[12—14] It was also reported that reduced glutathione (GSH) nonenzymatically reduces pentavalent methyl arsenicals to trivalent methyl arsenicals in water, thus resulting in the formation of $MMAs^{III}DG$ and $DMAs^{III}G$ *in vitro*.[15,16] Scott *et al.* reported that $MMAs^{III}DG$ and $DMAs^{III}G$ were produced *in vitro* by nonenzymatic reactions between $MMAs^V$ or $DMAs^V$ and GSH at molar ratios of $MMAs^V : GSH = 1 : 4$ or $DMAs^V : GSH = 1 : 3$.[15] Therefore, investigation of the *in vitro* cytolethality of arsenical–GSH mixtures is important in order to define the *in vivo* cytolethality of trivalent methyl arsenicals.

We previously reported that cellular GSH plays an important role in the cytolethality of arsenicals. Cellular GSH protects against the cytolethality of inorganic arsenicals and $MMAs^V$, but is required for $DMAs^V$-induced cytolethality.[8,17—22] On the other hand, the effect of exogenous GSH on the cytolethality of $MMAs^V$ and $DMAs^V$ is not clear. Some researchers have reported that exogenous GSH enhanced cytolethality of $MMAs^V$ and $DMAs^V$ *in vitro*, while others have

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found that exogenous GSH decreased them.[8,19—21,23—27] These contrasting results may depend on differences in the concentrations of arsenicals and GSH used in their experiments. In the present study, we observed the true effects of exogenous GSH on the cytolethality of $MMAs^V$ and $DMAs^V$ by investigating reactions between various concentrations of $MMAs^V$ or $DMAs^V$ and GSH. This study may thus provide important information on the cytolethalities of $MMAs^{III}DG$ and $DMAs^{III}G$.

MATERIALS AND METHODS

Chemicals $MMAs^V$ was purchased from Trichemical Co. (Yamanashi, Japan). $DMAs^V$ was purchased from Wako Pure Chemical Co. (Osaka, Japan). These arsenicals were recrystallized twice, and their purities were>99.9% as determined by GC-MS.[8] Endotoxin contamination of these arsenicals was not detected (<0.0000003%, wt/wt) using the endotoxin-specific limulus test (Seikagaku Co., Tokyo, Japan). L-Buthionine (*S,R*)-sulfoximine (BSO), reduced glutathione (GSH), oxidized glutathione (GSSG), L-cysteine (Cys), *N*-acetyl-L-cysteine (NAC) and 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Fetal bovine serum (FBS) was purchased from Thermo Electron Co. (Melbourne, Australia).

Cell Culture The TRL 1215 cell line is a rat epithelial liver cell line originally derived from the liver of 10-day old Fisher F344 rats.[28] TRL 1215 cells were cultured in William's E medium (Sigma) supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin G and 100 μg/ml streptomycin under a humidified atmosphere of 5% CO_2/95% air at 37 °C.

Assay for Cytolethality Cells were isolated by trypsinization, washed twice and resuspended in fresh medium. 2×10^4 cells/100 μl/well were plated on flat-bottomed 96-well tissue culture plates and allowed to adhere to the plate for 24 h, at which time the medium was removed and replaced with fresh medium containing the various test samples. Cells were then incubated with test samples for an additional 48 h. After incubation, cells were washed twice with warmed phosphate-buffered saline (pH 7.4) to remove non-adherent dead cells, and cell viability was determined by AlamarBlue assay, which is similar to MTT assay and measures metabolic integrity.[8] Briefly, after incubations with test samples and replacement with 100 μl/well fresh media, 10 μl/well AlamarBlue solution (Iwaki Grass Co., Chiba, Japan) was added directly to the 96-well plates, incubated for 4 h at 37 °C, and the absorbance at 570 nm (referenced to 600 nm) was measured by a microplate reader model 550 (Bio-Rad Laboratories, Hercules, CA, U.S.A.). Data are expressed as metabolite integrity using the values from control cells as 100%.

TLC TLC was performed with high performance TLC (HPTLC) plates silica gel 60 F 254 (Merck, Darmstadt, Germany) with a developing solvent of ethyl acetate : acetic acid : water (3 : 2: 1).[18] Iodide vapor was used for the detection of the various arsenicals.[18]

Arsenic Analysis TRL 1215 cells were grown in flat-bottomed 75-cm^2 tissue culture flask to confluence and exposed to test samples, including arsenicals. After the exposure, cells were isolated by trypsinization and rinsed twice with phosphate-buffered saline (pH 7.4). Cells were lysed with 1 ml of distilled water, and 3 ml of nitric acid and 1 ml of sulfuric acid were then added to the cell lysates. Cell lysates were heated at 240 °C until sulfur trioxide was visible. The digested solutions were neutralized with ammonium hydroxide, and distilled water was added to make a volume of 8 ml. One milliliter of hydrochloric acid, 0.5 ml of 20% ascorbic acid and 0.5 ml of 20% potassium iodide were then added to the solutions. The total amount of arsenic was analyzed by hydride generation coupled with atomic absorption spectrometry (AAS) using SpestraAA-220 (Varian Australia Pty Ltd., Mulgrave, Victoria, Australia).[29] The results are expressed as nanograms of arsenic per milligram of cellular protein determined by BCA protein assay (Pierce Co., Rockford, IL, U.S.A., with bovine serum albumin as a standard).

Statistical Analysis The data represent the mean± S.E.M., and statistical evaluations were performed by the Student's *t*-test or ANOVA followed by Dunnett's multiple comparison test as appropriate.[30] A value of $p<0.05$ was considered significant in all cases.

RESULTS

$DMAs^V$ Easily Combines with GSH in Water In order to determine the likelihood of DMAs–GSH conjugate production, 1 mM $DMAs^V$ was incubated with or without 1, 3, 5 or 10 mM GSH in distilled water for 1 h at 37 °C. After incubation, these mixtures were applied to an HPTLC plate and separated using ethyl acetate : acetic acid : water (3 : 2 : 1). Separated compounds were detected with iodide vapor. As shown in Fig. 1, GSH [lane 1, relative mobility (*Rf*)=0.33] and GSSG (lane 2, *Rf*=0.06) spots were detected with iodide vapor, but $DMAs^V$ was not detected under these experimental conditions (lane 3). The GSH spot did not appear when 1 mM $DMAs^V$ was incubated with 1 or 3 mM GSH, and a putative DMAs–GSH conjugate spot was detected at a different position from the GSH and GSSG spots (lanes 5—7, *Rf*= 0.49) after incubating 1 mM $DMAs^V$ with >3 mM GSH. The similar results were observed when $DMAs^V$ and GSH were reacted in phosphate buffer (pH=7.4) for 1 h at 37 °C (data

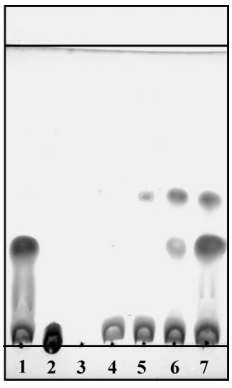

Fig. 1. $DMAs^V$ Easily Combines with GSH in Water

$DMAs^V$ (1 mM) was incubated with (1, 3, 5 or 10 mM) or without GSH in distilled water for 1 h at 37 °C. After the incubation, aliquots (25 μl) of these mixtures were spotted on HPTLC plate, developed with solvent of ethyl acetate : acetic acid : water (3 : 2 : 1), and the separated spots were detected with iodide vapor. Lane 1, GSH (10 mM) only; lane 2, GSSG (10 mM) only; lane 3, $DMAs^V$ (1 mM) only; lane 4, $DMAs^V$ (1 mM) plus GSH (1 mM); lane 5, $DMAs^V$ (1 mM) plus GSH (3 mM); lane 6, $DMAs^V$ (1 mM) plus GSH (5 mM); lane 7, $DMAs^V$ (1 mM) plus GSH (10 mM).

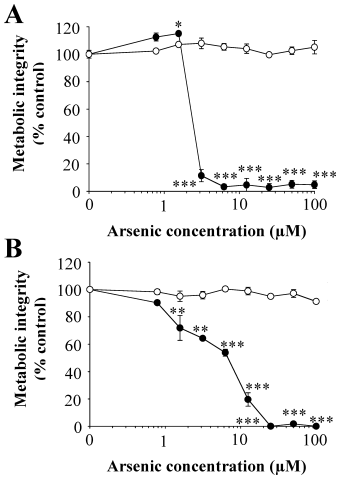

Fig. 2. Effect of Exogenous GSH on the Cytolethality of $MMAs^V$ or $DMAs^V$; the Mixture of High-Concentration $MMAs^V$ or $DMAs^V$ with GSH Showed Strong Cytolethality

Low (1—100 μM; open circle) or high (40 mM; closed circle) concentrations of $MMAs^V$ (A) or $DMAs^V$ (B) were pre-incubated with GSH at molar ratios of $MMAs^V$: GSH = 1 : 4 or $DMAs^V$: GSH = 1 : 3 in distilled water for 1 h at 37 °C. After the incubation, TRL 1215 cells were exposed to these arsenical–GSH mixtures at final arsenic concentrations until 100 μM for 48 h. Cellular viability was then assessed by AlamarBlue assay. Results are expressed as arithmetic mean ± S.E.M. of three separate experiments performed in triplicate (n=9). $*p<0.05$, in comparison to control cells incubated with medium alone. $**p<0.01$. $***p<0.001$.

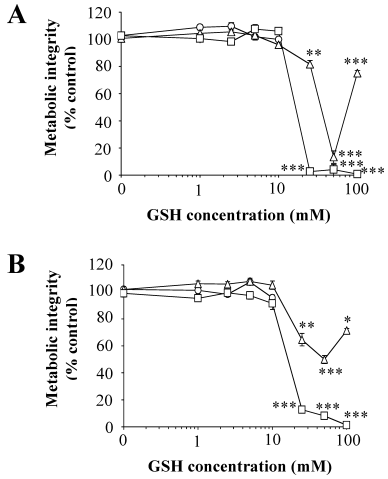

Fig. 3. Effect of Exogenous GSH on the Cytolethality of $MMAs^V$ or $DMAs^V$; mM Levels of $MMAs^V$ or $DMAs^V$ and ≥25 mM GSH Were Needed to Appear the Cytolethality of Arsenical–GSH Mixture

100 μM (open circle), 1 mM (open triangle) or 10 mM (open square) $MMAs^V$ (A) or $DMAs^V$ (B) was pre-incubated with various concentrations of GSH (1—100 mM; GSH/arsenicals ratios = 0.1—100) in distilled water for 1 h at 37 °C. After the incubation, TRL 1215 cells were exposed to these arsenical-GSH mixtures at a final arsenic concentration of 100 μM for 48 h. Cellular viability was then assessed by AlamarBlue assay. Results are expressed as arithmetic mean ± S.E.M. of three separate experiments performed in triplicate (n=9). $*p<0.05$, in comparison to control cells incubated with medium alone. $**p<0.01$. $***p<0.001$.

not shown).

We also examined the production of MMAs–GSH conjugate from $MMAs^V$ and GSH using the same HPTLC system. $MMAs^V$ was not detected under these experimental conditions. When $MMAs^V$ was incubated with GSH, the GSH spot disappeared and a putative MMAs–GSH conjugate spot was detected. This putative MMAs–GSH conjugate spot did not move from the starting point under these experimental conditions (data not shown).

Effect of Exogenous GSH on the Cytolethality of $MMAs^V$ or $DMAs^V$ Low (1—100 μM) or high (40 mM) concentrations of $MMAs^V$ or $DMAs^V$ were pre-incubated with GSH at molar ratios of $MMAs^V$: GSH = 1 : 4 or $DMAs^V$: GSH = 1 : 3 in distilled water for 1 h at 37 °C. TRL 1215 cells were exposed to these arsenical–GSH mixtures at final arsenic concentration of until 100 μM for 48 h, and cellular viability was then assessed. As shown in Fig. 2, the mixture of low-concentration $MMAs^V$ or $DMAs^V$ with GSH was not cytotoxic; however, the mixture of high-concentration $MMAs^V$ or $DMAs^V$ with GSH was strongly cytotoxic (LC_{50} values as As; mixture of 40 mM $MMAs^V$ with 160 mM GSH = 2.5 μM, mixture of 40 mM $DMAs^V$ with 120 mM GSH = 7.0 μM). The same concentrations (until 100 μM) of $MMAs^V$ or $DMAs^V$ alone were not cytotoxic. Exogenous GSH alone showed low cytolethality after 48 h of incubation with an LC_{50} value of 12 mM (data not shown).

Next, 100 μM, 1 mM or 10 mM $MMAs^V$ or $DMAs^V$ was pre-incubated with various concentrations of GSH (1—100 mM, GSH/arsenical ratios = 0.1—100) in distilled water for 1 h at 37 °C. TRL 1215 cells were then exposed to these arsenical-GSH mixtures at a final arsenic concentration of 100 μM for 48 h and cellular viability was assessed (Fig. 3). Concentration of exogenous GSH had no effect on the cytolethality of $MMAs^V$ and $DMAs^V$ when incubated with 100 μM $MMAs^V$ and $DMAs^V$; no cytotoxicity was noted. When 100 μM $MMAs^V$ or $DMAs^V$ was pre-incubated with 100 mM GSH, these arsenical–GSH mixtures were also not cytotoxic at a final arsenic concentration of 10 μM (data not shown).

When GSH was pre-incubated with 1 or 10 mM $MMAs^V$ and $DMAs^V$, GSH at <25 mM did not affect the cytolethality of $MMAs^V$ and $DMAs^V$, but GSH at ≥25 mM significantly increased them. When GSH was pre-incubated with 1 mM $MMAs^V$ or $DMAs^V$, GSH increased the cytolethality of $MMAs^V$ and $DMAs^V$ at 50 mM. In addition, 100 mM GSH increased the cytolethality of 1 mM $MMAs^V$ or $DMAs^V$, but its effect was lower than that of 50 mM GSH.

Effect of Exogenous GSH on Cellular Uptake of $MMAs^V$ or $DMAs^V$ $MMAs^V$ or $DMAs^V$ at 1 mM was pre-incubated with 5 mM (GSH/arsenical ratio = 5), 50 mM (GSH/arsenical ratio = 50) or 100 mM (GSH/arsenical ratio = 100) GSH in distilled water for 1 h at 37 °C. TRL 1215 cells were exposed to these arsenical–GSH mixtures at a final arsenic concentration of 100 μM for 48 h, and cellular arsenic contents were then measured. In the presence of exogenous GSH, cellular arsenic uptake of $MMAs^V$ were higher than that of $DMAs^V$. GSH at 5 or 100 mM did not affect cellular arsenic contents, while 50 mM GSH significantly increased them (Fig. 4).

Effects of DMPO, GSH, Cys, NAC, Serum or BSO on the Cytolethality and Cellular Uptake of MMAs– or DMAs–GSH Conjugates Various studies have suggested that arsenicals are ultimately toxic *via* the production of reactive oxygen species (ROS).[31] We investigated whether ROS participated in the cytolethality of MMAs– or DMAs–GSH conjugates. $MMAs^V$ or $DMAs^V$ at 40 mM was combined with GSH by pre-incubation with 160 mM or 120 mM GSH, respectively, in distilled water for 1 h at 37 °C. TRL 1215 cells were exposed to these methyl arsenical–GSH conjugates for 48 h in the presence or absence of 10 mM DMPO, a membrane-permeable ROS trapping reagent. DMPO at this con-

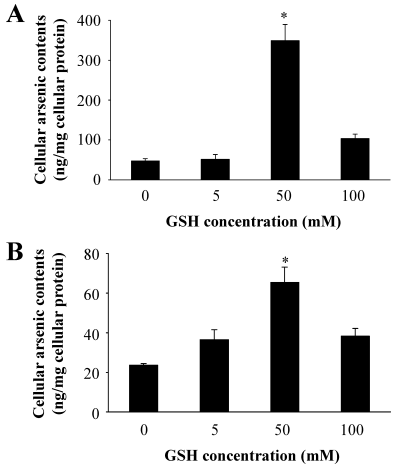

Fig. 4. Effect of Exogenous GSH on Cellular Uptake of $MMAs^V$ or $DMAs^V$

$MMAs^V$ (A) or $DMAs^V$ (B) at 1 mM was pre-incubated with 5 mM (GSH/arsenical ratio=5), 50 mM (GSH/arsenical ratio=50) or 100 mM (GSH/arsenical ratio=100) GSH in distilled water for 1 h at 37 °C. After the incubation, TRL 1215 cells were exposed to these arsenical–GSH mixtures at a final arsenic concentration of 100 μM for 48 h. Cellular arsenic contents were then measured by AAS. Results are expressed as arithmetic mean±S.E.M. of three separate experiments performed in triplicate (n=9). $*p<0.05$, in comparison to the cells exposed to 100 μM $MMAs^V$ or $DMAs^V$ alone.

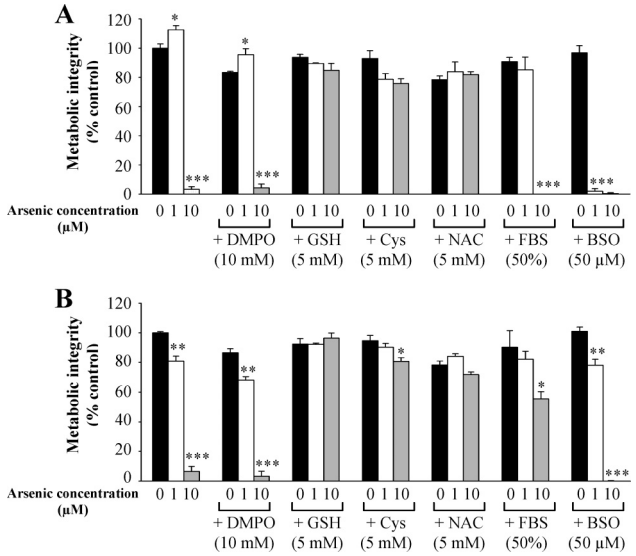

Fig. 5. Effects of DMPO, GSH, Cys, NAC, FBS or BSO on the Cytolethality of MMAs– or DMAs–GSH Conjugate

$MMAs^V$ or $DMAs^V$ at 40 mM was pre-incubated with 160 mM or 120 mM GSH, respectively, in distilled water for 1 h at 37 °C. TRL 1215 cells were pre-incubated with or without 50 μM BSO for 24 h, and were further exposed to MMAs– or DMAs–GSH conjugate at a final arsenic concentration of 0 (solid bars), 1 (open bars) or 10 μM (oblique bars) for 48 h in the presence or absence of 10 mM DMPO, 5 mM GSH, 5 mM Cys, 5 mM NAC, 50% FBS or 50 μM BSO. Cellular viability was then assessed by AlamarBlue assay. Results are expressed as arithmetic mean±S.E.M. of three separate experiments performed in triplicate (n=9). $*p<0.05$, in comparison to cells incubated with medium alone in the presence or absence of DMPO, GSH, Cys, NAC, FBS, or BSO. $**p<0.01$. $***p<0.001$.

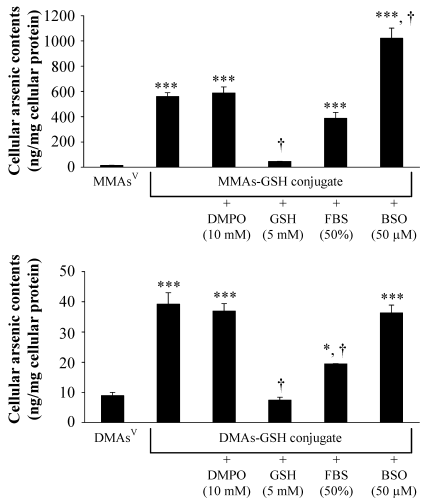

Fig. 6. Effects of DMPO, GSH, Cys, NAC, FBS or BSO on Cellular Arsenic Contents in the Cells Exposed to MMAs– or DMAs–GSH Conjugate

$MMAs^V$ or $DMAs^V$ at 40 mM was pre-incubated with 160 mM or 120 mM GSH, respectively, in distilled water for 1 h at 37 °C. TRL 1215 cells were pre-incubated with or without 50 μM BSO for 24 h, and were further exposed to MMAs– or DMAs–GSH conjugate at a final arsenic concentration of 10 μM for 48 h in the presence or absence of 10 mM DMPO, 5 mM GSH, 5 mM Cys, 5 mM NAC, 50% FBS or 50 μM BSO. The cellular arsenic contents were then measured by AAS. Results are expressed as arithmetic mean±S.E.M. of three separate experiments performed in triplicate (n=9). $*p<0.05$, in comparison to the cells exposed to 10 μM $MMAs^V$ or $DMAs^V$ alone. $***p<0.001$. $\dagger p<0.001$, in comparison to cells exposed to MMAs– or DMAs–GSH conjugate alone.

centration effectively scavenged cellular ROS and did not influence cell viability.[18,22] The addition of DMPO had no effect on the cytolethality or cellular uptake of MMAs– or DMAs–GSH conjugates (Figs. 5, 6).

We also investigated the effects of exogenous thiol agents, such as GSH, Cys and NAC, and serum (FBS) on the cytolethality of MMAs– or DMAs–GSH conjugates. TRL 1215 cells were exposed to MMAs– or DMAs–GSH conjugates for 48 h in the presence or absence of 5 mM GSH, 5 mM Cys, 5 mM NAC or 50% serum. Addition of GSH, Cys or NAC substantially reduced the cytolethality of the arsenical-GSH conjugates (Fig. 5). Exogenous GSH significantly decreased the cellular uptake of arsenical–GSH conjugates (Fig. 6). Addition of serum also significantly prevented both the cytolethality and cellular uptake of DMAs–GSH conjugate, but did not affect MMAs–GSH conjugate-induced cytolethality or cellular arsenic uptake (Figs. 5, 6).

We also observed the effects of GSH depletion on the cytolethality and cellular uptake of MMAs– or DMAs–GSH conjugates. TRL 1215 cells were exposed to MMAs– or DMAs–GSH conjugates for 48 h in the presence or absence of 50 μM BSO, a specific glutathione synthase inhibitor, and cell viability and cellular arsenic contents were then assessed. GSH depletion significantly enhanced both the cytolethality and cellular uptake of MMAs–GSH conjugate, but had no effect on the cytolethality or cellular uptake of DMAs–GSH conjugate (Figs. 5, 6).

DISCUSSION

Arsenic intoxication occurs widely through the consumption of contaminated well water or foods containing inorganic arsenicals.[2] In many mammalian species, inorganic arsenate is first reduced to arsenite and is subsequently methylated to $MMAs^V$ and $DMAs^V$. The methylation of inorganic arsenicals was initially thought to be a detoxification process because the toxicity of $MMAs^V$ and $DMAs^V$ is substantially lower than that of inorganic arsenicals.[7,8] However, it was recently reported that toxic $MMAs^{III}$ and $DMAs^{III}$ might be produced through the methylation of inorganic arsenicals,[9,10] and that arsenical–glutathione conjugates, such as $MMAs^{III}DG$ and $DMAs^{III}G$, might be generated *in vivo*.[12—14]

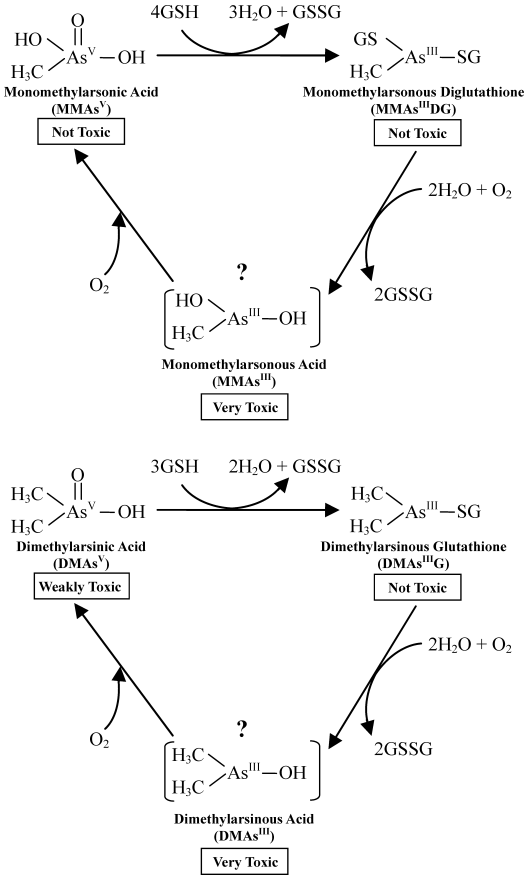

Fig. 7. The Putative Nonenzymatic Chemical Reactions of Monomethylarsenic or Dimethylarsenic Compounds with GSH

$MMAs^{III}DG$ and $DMAs^{III}G$ are also produced by nonenzymatic reactions between $MMAs^{V}$ or $DMAs^{V}$ and GSH *in vitro*.[15,16] Studying the effects of exogenous GSH on the cytolethality of $MMAs^{V}$ and $DMAs^{V}$ may thus indicate the *in vivo* cytolethality of $MMAs^{III}$ and $DMAs^{III}$.

Scott *et al.* reported that $MMAs^{V}$ or $DMAs^{V}$ combined with GSH at molar ratios of $MMAs^{V}$: GSH = 1 : 4 or $DMAs^{V}$: GSH = 1 : 3, and that $MMAs^{III}DG$ or $DMAs^{III}G$ was formed *in vitro* (Fig. 7).[15] When $MMAs^{V}$ or $DMAs^{V}$ was pre-incubated with GSH at molar ratios of $MMAs^{V}$: GSH = 1 : 4 or $DMAs^{V}$: GSH = 1 : 3, $MMAs^{V}$ or $DMAs^{V}$ at μM levels mixed with GSH was not cytotoxic, but $MMAs^{V}$ or $DMAs^{V}$ at mM levels mixed with GSH was strongly cytotoxic at the same arsenic concentrations (Fig. 2). These results indicate that the formation of cytotoxic $MMAs^{III}DG$ and $DMAs^{III}G$ depends on arsenical and GSH concentrations. In this study, we confirmed that DMAs–GSH conjugate, may be $DMAs^{III}G$, was formed during incubation of 1 mM $DMAs^{V}$ with >3 mM GSH (Fig. 1), and that MMAs–GSH conjugate, may be $MMAs^{III}DG$, was formed during incubation of 1 mM $MMAs^{V}$ with >4 mM GSH (data not shown).

It was suggested that toxic trivalent dimethylarsine gas might be formed in the reaction between $DMAs^{V}$ and GSH in cell culture medium.[23] In a previous study, we did not detect any gas when $DMAs^{V}$ was mixed with GSH, irrespective of concentration ratio, nor was any gas detected when GSH was mixed with $MMAs^{V}$.[19] Thus, significant cytolethality of $MMAs^{V}$– or $DMAs^{V}$–GSH mixture may not be caused by toxic gas generation, but rather may depend on arsenical–GSH conjugate generation. We also observed the effects of various concentrations of GSH on the cytolethality of $MMAs^{V}$ or $DMAs^{V}$. As shown in Fig. 3, arsenical–GSH mixtures showed cytolethality only after the incubating mM levels of $MMAs^{V}$ or $DMAs^{V}$ with ≥25 mM GSH. These findings indicate that mM levels of $MMAs^{V}$ or $DMAs^{V}$ and ≥25 mM GSH are needed to form arsenical–GSH conjugates that show significant cytolethality. It has been reported that plasma GSH concentrations remain at μM levels in healthy humans.[32,33] It has been also demonstrated that arsenic concentrations in the plasma of chronic arsenic poisoning patients is at the μM level.[34] Therefore, large amounts of cytotoxic $MMAs^{III}DG$ and $DMAs^{III}G$ are probably not produced nonenzymatically at *in vivo* levels of arsenicals and GSH.

$MMAs^{III}$ and $DMAs^{III}$ have been reported to be highly cytotoxic and genotoxic in previous studies using synthetic trivalent methyl arsenicals, such as $MMAs^{III}O$ and $DMAs^{III}I$.[11] However, the reason for the strong cytolethality of trivalent methyl arsenicals has not been thoroughly investigated. In this study, it was suggested that exogenous GSH increased the cytolethality of $MMAs^{V}$ and $DMAs^{V}$ by at least partially increasing cellular arsenic uptake (Figs. 4, 6). A membrane-permeable ROS trapping reagent, DMPO, had no effect on either cytolethality or cellular arsenic contents induced by arsenical–GSH conjugate exposure, and thus a role for ROS is unlikely (Figs. 5, 6). Interestingly, exogenous thiol agents, such as GSH, Cys and NAC, greatly decreased both the cytolethality and cellular arsenic contents induced by arsenical–GSH conjugate exposure (Figs. 5, 6). It is generally believed that the large GSH molecule is not transported efficiently into the cells.[35] Therefore, these thiol agents maintain the form of $MMAs^{III}DG$ and $DMAs^{III}G$ or combine with new arsenicals, and these conjugates cannot subsequently be transported into cells. As shown in Figs. 3, 4, 50 mM GSH increased both the cytolethality and cellular uptake of $MMAs^{V}$ and $DMAs^{V}$, and the effect was potent than with 100 mM GSH. Some GSH was thought to have remained unreacted when 100 mM GSH was incubated with arsenicals. Over concentrations of GSH might maintain the form of impermeable $MMAs^{III}DG$ and $DMAs^{III}G$. When $MMAs^{III}DG$ and $DMAs^{III}G$ show significant cytolethality, it may be that they separated into trivalent methyl arsenicals and glutathione, probably becoming $MMAs^{III}(OH)_2$ and $DMAs^{III}OH$, before being transported into cells (Fig. 7). In addition, normal serum (FBS) significantly reduced both the cytolethality and cellular arsenic contents induced by $DMAs^{III}G$ exposure, although it did not affect either the cytolethality or cellular arsenic contents induced by $MMAs^{III}DG$ exposure (Figs. 5, 6). The reason for serum preventing the cytolethality of only $DMAs^{III}G$ is unclear, but it is indicated that serum specifically reduced the cytolethality of $DMAs^{III}G$ by (1) maintaining the form of $DMAs^{III}G$, (2) combining protein thiols in serum with $DMAs^{III}$, or (3) facilitating the oxidation of $DMAs^{III}$. Significant cytolethality of $MMAs^{III}DG$ and $DMAs^{III}G$ may not be observed *in vivo* because extracellular thiol agents and normal serum greatly block the cytolethality of arsenical–GSH conjugates.

We also investigated the effect of cellular GSH depletion on the cytolethality of arsenical–GSH conjugates. GSH depletion significantly increased the cytolethality and cellular arsenic contents induced by $MMAs^{III}DG$ exposure (Figs. 5,

6). It was recently reported that conjugation of $MMAs^{V}$ with GSH was important for its excretion *via* several transporters.[12,36,37] Thus, $MMAs^{III}DG$ may be converted to $MMAs^{III}(OH)_2$ and subsequently transported into cells, after which it may recombine with cellular GSH and be pumped out of cells. However, GSH depletion did not affect the cytolethality and cellular arsenic contents induced by $DMAs^{III}G$ exposure (Figs. 5, 6). Therefore, the mechanism of $DMAs^{III}$-induced cytolethality may be different from that of $MMAs^{III}$-induced cytolethality. Further research is needed to determine the mechanism of trivalent methyl arsenical-induced cytolethality. We previously reported that there were substantial differences between the mechanism of $DMAs^{V}$-induced cytolethality and that of $MMAs^{V}$-induced cytolethality, and cellular GSH played different roles in the cytolethality of $MMAs^{V}$ and $DMAs^{V}$.[8,17—22]

GSH may be a key molecule in preventing or inducing arsenic cytolethality. GSH nonenzymatically combines with $MMAs^{V}$ and $DMAs^{V}$, resulting in the generation of $MMAs^{III}DG$ and $DMAs^{III}G$.[15,16] The present results suggested that arsenical and GSH concentrations greatly affect the formation and cytolethality of arsenical–GSH conjugates. Arsenical and GSH concentrations higher than mM levels are needed to form arsenical–GSH conjugates with significant cytolethality. Some previous studies have shown that exogenous GSH increased cytolethality of $MMAs^{V}$ and $DMAs^{V}$ *in vitro*, while other studies have suggested that exogenous GSH decreased them. These contrasting results must depend on differences in the concentrations of methylated arsenicals and GSH used in their experiments. Arsenical–GSH conjugates may exert their cytolethality by separating into trivalent methyl arsenicals and glutathione, and being transported into cells. On the other hand, at *in vivo* levels (μM levels), arsenicals and GSH may not nonenzymatically produce sufficient amounts of cytotoxic arsenical–GSH conjugates. Furthermore, extracellular thiol agents and normal serum significantly decrease the cytolethality of arsenical–GSH conjugates by preventing cellular arsenic uptake. Therefore, the significant cytolethalities of $MMAs^{III}DG$ and $DMAs^{III}G$ may never manifest in the normal human body. Further research will be required in order to determine the role of GSH and methylation in the cytolethality of arsenicals in chronic arsenic poisoning patients who regularly ingest arsenic-contaminated well water and/or in acute promyelocytic leukemia patients who are injected with arsenite as a chemotherapeutic agent.

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